

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 37/38	A1	(11) International Publication Number: WO 92/04913 (43) International Publication Date: 2 April 1992 (02.04.92)
(21) International Application Number: PCT/US91/06487 (22) International Filing Date: 13 September 1991 (13.09.91) (30) Priority data: 582,642 13 September 1990 (13.09.90) US (71) Applicant: CHILDREN'S HOSPITAL MEDICAL CENTER OF NORTHERN CALIFORNIA [US/US]; 747 52nd Street, Oakland, CA 94609 (US). (72) Inventor: PERRINE, Susan, P. ; 2682 Longview Drive, Richmond, CA 94806 (US). (74) Agents: SUYAT, Reginald, J. et al.; Heller, Ehrman, White & McAuliffe, 333 Bush Street, San Francisco, CA 94104-2878 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHOD FOR INCREASING RED BLOOD CELL PRODUCTION BY TREATMENT WITH ACTIVIN OR ACTIVIN-RELATED PEPTIDES (57) Abstract A method is provided for increasing red blood cell production. The method is particularly adapted for ameliorating the clinical symptoms of anemia introducing into the subject an effective amount of activin, inhibin, an inhibin chain or mixtures thereof.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire---	KR	Republic of Korea	SU ⁺	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE*	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

⁺ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

-1-

METHOD FOR INCREASING RED BLOOD CELL PRODUCTION BY
TREATMENT WITH ACTIVIN OR ACTIVIN-RELATED PEPTIDES

The present invention is directed to a method for increasing the production of red blood cells. In particular, the present invention is directed to a method for treatment of diseases or conditions associated with deficient or defective red blood cells by introducing activin to a mammal, such as, for treatment of anemia. The present invention is particularly directed to treatment of anemias which are not effectively treated by erythropoietin, such as AZT-induced anemia.

BACKGROUND OF THE INVENTION

Anemias and other diseases associated with lowered or defective red blood cells may be treated with erythropoietin. However, some anemias, such as anemia induced by AZT intake for therapy against HIV infection, is not effectively counteracted with erythropoietin. Also, red blood cell enhancement is desirable pre-operatively for autologous transfusions, as well as in conditions causing red cell failure states, when erythropoietin elevation in an attempt to raise red blood cell level is usually to no avail. Red blood cell enhancement is also often needed in conjunction with chemotherapy or in recovery from bone marrow transplant. Hydroxyurea, frequently used in anemia treatment, is too toxic for some patients.

-2-

It is thus an object of the present invention to provide a method for increasing production of red blood cells by administering activin or an activin-related peptide, particularly in individuals with anemias or in need of enhanced red blood cell levels.

Activin, a hormone, sometimes also referred to as erythroid differentiation factor (EDF) or follicle-stimulating hormone releasing protein (FRP), is a homodimer consisting of either two β_A subunits of inhibin (Activin A), two β_B subunits of inhibin (Activin B), or a subunit each of β_A and β_B (Activin AB). Inhibin is another hormone which, among other effects, suppresses secretion of FSH (follicle-stimulating hormone) from the pituitary gland. Inhibin is a protein consisting of α and β_A subunits linked by disulfide bonds. Activin is present, in analogous forms, in mammals and have been reported, for instance, in human, porcine, and bovine follicular fluid. Porcine inhibin has been purified and sequenced from porcine follicular fluid as described in U.S. Patent 4,740,587. The DNA encoding the prepro inhibin α and β chains of porcine or human inhibin has been isolated, ligated into expression vectors and expressed in mammalian culture. See European Patent Application No. 222,491, published May 20, 1987. Activin A has been shown to induce hemoglobin accumulation in a human erythroleukaemic cell line and to induce the proliferation of erythroid progenitor cells in human bone marrow culture. See Yu, *et al.*, *Nature*, 330, 765 (December 24, 1987). The structures and isolation of activin have been reported by several groups in the literature. See Vale, *et al.*, *Nature*, 321: 776 (1986); Ling, *et al.*, *Nature*, 321: 779 (1986); Ito, *et al.*, *Biochem. Biophys. Res. Comm.*, 142, 1095 (1987); Tsuji, *et al.*, *Biotech. Bioeng.*, 31, 675 (1988); Shibata, *et al.*, *Biochem. Biophys. Res. Comm.*, 146, 187 (1987).

-3-

SUMMARY OF THE INVENTION

The present invention provides a method for enhancing red blood cell production in vivo or in vitro comprising the step of introducing to a mammal or to erythroid culture, a compound selected from the group consisting of activin, inhibin, an inhibin chain and mixtures thereof, in an effective amount sufficient to increase red blood cell production. The method according to the present invention is particularly useful for ameliorating in humans the clinical effects of anemias.

DESCRIPTION OF THE INVENTION

The present invention provides a method for increasing the production in vivo or in vitro of red blood cells.

15 In accordance with the present invention, activin, inhibin, in any of their analogous mammalian forms, or mixtures of these are introduced to the subject (or culture) receiving enhancement of red blood cell production, such as in the case of anemic subjects.

20 As used herein, the term "biological sample" means any cells or body fluid from a mammal that can be diagnosed, including blood erythroid progenitors.

It is also intended that variants and single chains of activin or inhibin will be utilized alone or in mixtures with each other, or with activin and/or inhibin. By the terms "activin" and "inhibin" it is meant the dimers of α and β -chains of inhibin, prepro forms, and their prodomains, together with glycosylation and/or amino acid sequence variants thereof. The precursor may be used with or without the mature protein, and, after cleavage from the mature protein, may be non-covalently associated with the mature protein. By the term "inhibin chain" it is meant to include, but not to be limited to, the α and β chains of inhibin, as well as their prepro

-4-

forms and their prodomains, together with glycosylation and/or amino acid sequence variants of each chain thereof.

Generally, amino acid sequence variants will be
5 substantially homologous with the relevant portion of the porcine or human α or β chain sequences set forth in the aforementioned European Patent Application 222,491, which is incorporated herein by reference in its entirety.

10 Substantially homologous means that greater than about 60% of the primary amino acid sequence of the homologous polypeptide corresponds to the sequence of the porcine or human chain when aligned in order to maximize the number of amino acid residue matches between the two
15 proteins. Alignment to maximize matches of residue includes shifting the amino and/or carboxyl terminus, introducing gaps as required and/or deleting residues present as inserts in the candidate. Typically, amino acid sequences variants will be greater than about 70%
20 homologous with the corresponding native sequences.

Variants that are not hormonally-active fall within the scope of this invention, and include polypeptides that may or may not be substantially homologous with either a mature inhibin chain or prodomain sequence, but which
25 are (1) immunologically cross-reactive with antibodies raised against the native counterpart or (2) capable of competing with such native counterpart polypeptides for cell surface receptor binding. Hormonally inactive variants are produced by the recombinant or organic
30 synthetic preparation of fragments, in particular the isolated β chains of inhibin, or by introducing amino acid sequence variations so that the molecules no longer demonstrate hormonal activity as defined above.

-5-

Immunological or receptor cross-reactivity means that the candidate polypeptide is capable of competitively inhibiting the binding of the hormonally-active analogue to its receptor and/or to polyclonal antisera raised
5 against the hormonally-active analogue. Such antisera are prepared in conventional fashion by injecting goats or rabbits S.C. with the hormonally-active analogue or derivative in complete Freund's adjuvant, followed by booster intraperitoneal or S.C. injections in incomplete
10 Freund's.

The variants of inhibin include the pro and/or prepro sequences of the inhibin α or β chain precursors, or their immunologically or biologically active fragments, substantially free of the corresponding mature inhibin
15 chains. The sequences for porcine and human inhibin are known, for example, as published in European Patent Application 222,491. The prepro sequence for the porcine α subunit precursor is the polypeptide comprised by residues 1 to about 230, while the β_A subunit pro
20 sequence is comprised by residues 1 to about 308. These sequences encompass prodomain sequences.

The intact isolated prepro or prodomain β_A , β_B or α sequences are best synthesized in recombinant cell culture and the individual subcomponent domains are
25 synthesized by routine methods of organic chemistry or by recombinant cell culture, for example as described in European Patent Application 222,491.

While the site for introducing a sequence variation is predetermined, it is unnecessary that the mutation per se
30 be predetermined. For example, in order to optimize the performance of mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed inhibin mutants screened for the optimal combination of desired activity. Techniques

-6-

for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis.

Mutagenesis is conducted by making amino acid
5 insertions, usually on the order of about from 1 to 10 amino acid residues, or deletions of about from 1 to 30 residues. Substitutions, deletions, insertions or any subcombination may be combined to arrive at a final construct. Preferably, however, only substitution
10 mutagenesis is conducted. Obviously, the mutations in the encoding DNA must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure.

15 Covalent modifications of inhibin, activin or prodomains are included within the scope hereof and include covalent or aggregative conjugates with other chemical moieties. Covalent derivatives are prepared by linkage of functionalities to groups which are found in the
20 inhibin amino acid side chains or at the N- or C-termini, by means known in the art. For example, these derivatives will include: aliphatic esters or amides of the carboxyl terminus or residues containing carboxyl side chains, e.g., aspartyl residues; O-acyl derivatives
25 of hydroxyl group-containing residues such as seryl or alanyl; and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g. lysine or arginine. The acyl group is selected from the group of alkyl-moieties (including C3 to C10 normal
30 alkyl), thereby forming alkanoyl species, and carbocyclic or heterocyclic compounds, thereby forming aroyl species. The reactive groups preferably are difunctional compounds known per se for use in cross-linking proteins to insoluble matrices through reactive
35 side groups, e.g. m-Maleimidobenzoyl-N-hydroxy

-7-

succinimide ester. Preferred derivatization sites are at histidine residues.

All of these variants of activin and inhibin, as well as inhibin itself, are intended to be within the scope
5 of the term "activin-related peptides."

The method used to introduce the compound will be any convenient method normally used to introduce pharmaceuticals into the bloodstream, such as by injection, bolus, infusion, and the like. Parenteral
10 administration may also be utilized.

The exact size of an effective dose of a compound according to the method of the present invention will depend on a number of factors, including the particular recipient and the severity of condition; thus the route
15 of administration will be ultimately at the discretion of the attendant physician. The diseases or conditions which may be treated include, but are not limited to, anemias, including AZT-induced anemia and chemotherapy-induced anemia, Diamond-Blackfan anemia, and the like.
20 The treatment may also be used in non-disease related conditions, such as during patient recovery after bone-marrow transplant, or to induce a pre-operative boost in red blood cells preparatory to autologous transfusions.

25 While it is possible to utilize the compounds in vivo per se, it is preferable to present them as a pharmaceutical formulation preparation. The formulation of the present invention comprises a compound as previously described together with one or more
30 acceptable carriers therefor and, optionally other therapeutic ingredients. The carriers must be acceptable in the sense of being compatible with other

-8-

ingredients of the formulation and not deleterious to the recipient.

Activin or an activin-related peptide may be administered to the patient by any suitable technique, including parenteral, sublingual, topical intrapulmonary, and intranasal administration. The specific route of administration will depend, e.g., on the type of therapy required. Examples of parenteral administration include intramuscular, subcutaneous, intravenous, intraarterial, and intraperitoneal administration.

The compositions to be used in the therapy will be formulated and dosed in a fashion consistent with good medical practice taking into account the clinical condition of the individual patient, the cause of the condition in need of therapy, the site of delivery of the composition, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" for purposes herein is thus determined by such considerations.

As a general proposition, the total pharmaceutically effective amount of the activin and/or activin-related peptide administered parenterally per dose will be in the range of about 50 μ g/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose is the result obtained, as measured by increase in red blood cell production, which may be measured, for example by in vitro analysis of erythroids of the patient evidencing increase in the number of colonies/culture and/or the number of cells/colony after treatment.

-9-

The composition herein is also suitably administered by sustained release systems. Suitable examples of sustained release compositions include semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules. Sustained release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (U. Sidman, et al., Biopolymers, 22, 547-556 (1983)), poly(2-hydroxyethyl methacrylate) (R. Langer, et al., J. Biomed. Mater. Res., 15: 167-277 (1981), and R. Langer, Chem. Tech. 12:: 98-105 (1982)), ethylene vinyl acetate (R. Langer, et al., Id.) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Sustained release compositions also include liposomally entrapped activin or inhibin or a mixture thereof. Such compositions are prepared by methods known per se: DE 3,218,121; Epstein, et al., Proc. Natl. Acad. Sci. U.S.A., 82: 3688-3692 (1985); Hwang, et al., Proc. Natl. Acad. Sci. U.S.A. 77: 4030-4034; EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appln. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal therapy.

For parenteral administration, the activin or activin-related peptide is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion) with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents

-10-

and other compounds that are known to be deleterious to polypeptides.

Generally, the formulations are prepared by contacting the activin or inhibin uniformly and intimately with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes. Generally, the carrier can contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives, as well as low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose or dextrans, chelating agents such as EDTA, or other excipients. The activin is typically formulated into such vehicles at a concentration of about 10/ μ g/ml to 100 μ g/ml at physiological pH.

Activin for use in therapeutic administration must be sterile. Sterility is readily accomplished by sterile filtration through (e.g., 0.2 micron) membranes. Activin B ordinarily will be stored in unit or multidose containers, for example, sealed ampoules or vials, as an aqueous solution, as it is highly stable to thermal and oxidative denaturation. Lyophilized formulations for reconstitution are also acceptable.

Preferred unit dosage formulations are those containing a daily dose or a unit daily subdose, or an appropriate fraction thereof.

-11-

As further application of the compounds according to the present method, they may be added in vitro to cell cultures taken from patients and the increase in the amount of red blood cell production measured to
5 determine the potential efficacy of further treatment for the disorders. The compounds may be thus used in vitro in cell cultures from patients to determine whether further addition of one of the compounds would result in continued increase or maintenance of red blood
10 cell production.

The frequency and dosages of administration of the above compounds will depend upon when the compound is introduced, whether the subject is a fetus, infant or adult, the size and weight of the subject, the condition
15 of the patient, and the like. Generally, injections or activin and/or activin-related peptide beginning at a dosage of about 50 μ g/kg-10 mg/kg; and often as low as 50 μ g/kg-100 μ g/kg body weight per day during gestation, particularly prior to the thirty-second week of
20 gestation in humans, will delay the γ to β switching. Dosages; up to about 10 mg/kg/day may be utilized at the discretion of the physician.

The method according to the present invention may be utilized in vivo, or in vitro as a diagnostic test by
25 measuring the increase in red blood cell production in the culture as compared to a control sample cultured in absence of the activin, inhibin, or inhibin chain. For the in vitro test erythroid cultures, such as that obtained from cord blood mononuclear cells in Iscove's
30 Modified Dulbcco's Medium with 0.9% methylcellulose, may be used as described by Stamatoyannopoulos et al., Blood, 54, 440-450 (1979) and Friedman et al., J. Clin. Invest., 75, 1359-1368 (1985).

-12-

The following examples are provided by way of illustration, however, the invention is not intended to be limited in any manner thereby.

EXAMPLE

- 5 Recombinantly produced human activin (produced as described in EP Publication No. 222,491, supra.) was used to test effect on cell colony growth in erythroid cultures from cord blood of normal infants. Samples were prepared containing 100 nanograms/ml activin and
10 control. The results below show that activin consistently enhanced red blood cell production.

Effect on Colony GrowthNumber of ColoniesCells/Colony

15

<u>Test No.</u>	<u>Control</u>	<u>Activin</u>	<u>Control</u>	<u>Activin</u>
1	21	30	28×10^3	35.6×10^3
2	25	43		
3	20	48		
4	20	50	2.6×10^3	16.8×10^3

-13-

WHAT IS CLAIMED IS:

1. A method for increasing red blood cell production in a mammal comprising the step of introducing to said mammal a compound selected from the group consisting of
5 activin, inhibin, an inhibin chain, and derivatives and mixtures thereof, in an amount effective to increase red blood cell production.
2. A method according to Claim 1 wherein said compound is selected from the group consisting of analogous
10 mammalian forms of inhibin and activin, inhibin α chain, inhibin β chain, prepro inhibin α chain, prepro inhibin β chain, an amino acid sequence variant of inhibin α chain, an amino acid sequence variant of inhibit α chain, an amino acid sequence variant of inhibit β
15 chain, pro inhibin α chain and pro inhibin β chain.
3. A method according to Claim 1 wherein said mammal is anemic.
4. A method according to Claim 1 wherein said compound comprises human activin.
- 20 5. A method according to Claim 1 wherein said compound comprises human inhibin.
6. A method according to Claim 1 wherein said compound comprises porcine activin.
7. A method according to Claim 1 wherein said compound
25 comprises porcine inhibin.
8. A method for ameliorating anemia in a mammal comprising the step of introducing activin to the mammal, in an amount effective to increase production of red blood cells.

-14-

9. A method for ameliorating anemia in a mammal comprising the step of administering inhibin or an inhibin chain to the mammal, in an amount effective to increase production of red blood cells.
- 5 10. A method for ameliorating anemia in a mammal comprising the step of administering a mixture of activin and inhibin or an inhibin chain to the mammal, in an amount effective to increase production of red blood cells.
- 10 11. A method according to Claim 1 wherein the introducing is conducted by administering the activin into the bloodstream of said mammal.
12. A method according to Claim 1 wherein the introducing is conducted by administering the inhibin
15 or inhibin chain in said mammal.
13. A method according to Claim 1 wherein the introducing is conducted by administering said mixture to said mammal.
14. A method according to Claim 8 wherein said activin
20 is an analogous mammalian form of activin, a precursor of activin, or a complex of mature activin and its precursor.
15. A method according to Claim 14 wherein the mammalian form of activin is porcine or human activin.
- 25 16. A method according to Claim 9 wherein said inhibin or inhibin chain is selected from the group consisting of analogous mammalian forms of inhibin, inhibin α -chain, inhibin β -chain, prepro inhibin α -chain, prepro inhibin β -chain, pro inhibin α -chain, pro inhibin

-15-

β -chain, the precursor of inhibin, and a complex of mature inhibin and its precursor.

17. A method according to Claim 16 wherein said inhibin is porcine or human inhibin.

5 18. A method according to Claim 8 wherein the red blood cell production is monitored by adding to an in vitro cell culture from the mammal an effective amount of said activin to determine if additional treatment is needed.

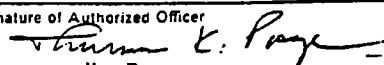
10 19. A method according to Claim 9 wherein the red blood cell production is monitored by adding to an in vitro cell culture from the mammal an effective amount of said inhibin or inhibin chain to determine if additional treatment is needed.

15 20. A method according to Claim 10 wherein the red blood cell production is monitored by adding to an in vitro cell culture from the mammal an effective amount of said mixture to determine if additional treatment is needed.

INTERNATIONAL SEARCH REPORT

PCT/US91/06487

International Application No.

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 37/38 U.S.CL. 514/12		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	514/2, 8, 12, 21, 814, 815; 530/350, 397	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	JP, A, 2-108627 (AJINOMOTO KK) 20 APRIL 1990 See the abstract.	1-4,6,8,11,14 & 15 18
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 165, 29 DECEMBER 1989 (SHIOZAKI), "In vivo treatment with Erythroid Differentiation Factor (EDF/Activin A) increases erythroid precursors (CFU-E and BFU-E) in mice", pages 1155-1161. See the abstract.	1-4,6,8,11,14 & 15
Y	Nature, Volume 330, 31 DECEMBER 1987 (YU), "Importance of FSH-releasing protein and inhibin in erythrodifferentiation", pages 765-767.	1-20
P,X	US, A, 5,032,507 (YU) 16 JULY 1991. See the abstract; column 2, line 24, column 3, lines 20-30, 50-54 and column 8, line 4.	1-4,6-8,11,14,15 5,9-10,12-13. 16-20
P,X	US, A, 4,997,815 (PERRINE) 05 MARCH 1991. See the abstract and claims.	1-20
<p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
26 November 1991	22 JAN 1992	
International Searching Authority	Signature of Authorized Officer	
ISA/US	 Thurman K. Page	

CONTINUED VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

II. Active agent species.

- a) activin, activin chain and derivatives in claims 1, 2, 4, 6, 8, 11, 14 and 15 of method Ia and in claim 18 of method Ib.
- b) inhibin, inhibin chain and derivatives in claims 1, 2, 5, 7, 9, 12, 16 and 17 of method Ia and in claim 19 of method Ib.
- c) mixture of activin, inhibin or derivatives in claims 1, 2, 10 and 13 of method Ia and in claim 20 of method Ib.

The above stated species are patentably distinct in that they are not connected in design or operation.